Dentine decalcification and smear layer removal by different ethylenediaminetetraacetic acid and 1-hydroxyethane-1,1-diphosphonic acid species

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Abstract: AIM To compare solutions of di- and tetrasodium ethylenediaminetetraacetic acid (EDTA) and 1-hydroxyethane-1,1-diphosphonic acid (HEDP) regarding their ability to solubilize calcium from dentine and remove smear layer. METHODOLOGY Solutions with a molarity corresponding to that of 17% Na EDTA (pH adjusted to 8.5) were prepared by dissolving Na and Na salts of HEDP (etidronate), or Na EDTA in deionized water. Standardized root dentine discs covered by a smear layer were prepared from human third molars. These discs (n = 10 per group) were immersed in test solutions or phosphate-buffered saline for 1 min. The dissolved Ca was determined by atomic absorption spectroscopy, apparently opened dentinal tubules by laser scanning microscopy and automated image analysis. Ca values were compared between groups by parametric, tubular areas by nonparametric methods, = 0.05. RESULTS Solutions prepared from the tetrasodium salts were alkaline (pH 11.3-11.4), whilst counterparts made from the disodium salts were acidic. The EDTA solutions dissolved more calcium than the HEDP counterparts ($P < 0.05$); solutions prepared with the disodium salts dissolved more calcium than those made from the tetrasodium salts ($P < 0.05$). There was a high correlation between dissolved calcium and the apparently opened tubular areas (Spearman's = 0.81). Differences between groups regarding opened tubules were similar to those observed regarding the Ca values, with a slightly reduced discerning power due to high variance. CONCLUSION Calcium chelation and thus smear layer removal by EDTA and HEDP are influenced by pH.

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Dentine decalcification and smear layer removal by different EDTA and HEDP species

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Running head: pH effect on HEDP and EDTA

Keywords: 1-hydroxyethane-1,1-diphosphonic acid, HEDP, etidronic acid, pH, smear layer

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Abstract

Aim To compare solutions of di- and tetrasodium EDTA and HEDP regarding their ability to solubilize calcium from dentine and remove smear layer.

Methodology Solutions with a molarity corresponding to that of 17% Na_2EDTA (pH adjusted to 8.5) were prepared by dissolving Na_2 and Na_4 salts of HEDP (etidronate), or Na_4EDTA in deionized water. Standardized root dentine disks covered by a smear layer were prepared from human third molars. These disks (n = 10 per group) were immersed in test solutions or phosphate-buffered saline for 1 min. The dissolved Ca^{2+} was determined by atomic absorption spectroscopy, apparently opened dentinal tubules by laser scanning microscopy and automated image analysis. Ca^{2+} values were compared between groups by parametric, tubular areas by non-parametric methods, alpha = 0.05.

Results Solutions prepared from the tetrasodium salts were alkaline (pH 11.3 - 11.4), while counterparts made from the disodium salts were acidic. The EDTA solutions dissolved more calcium than the HEDP counterparts ($P < 0.05$); solutions prepared with the disodium salts dissolved more calcium than those made from the tetrasodium salts ($P < 0.05$). There was a high correlation between dissolved calcium and the apparently opened tubular areas (Spearman's rho = 0.81). Differences between groups regarding opened tubules were similar to those observed regarding the Ca^{2+} values, with a slightly reduced discerning power due to high variance.

Conclusion Calcium chelation and thus smear layer removal by EDTA and HEDP are influenced by pH.

Keywords: 1-hydroxyethane-1,1-diphosphonic acid, HEDP, etidronic acid, pH, smear layer

Introduction

Decalcifying agents are used in the context of root canal instrumentation to remove the smear layer and reduce the amount of accumulated hard tissue debris (Hülsmann *et al.* 2003). Recent studies on the compatibility of calcium chelating (sequestering) agents with sodium hypochlorite have highlighted the possibility of using salts of ethylenediaminetetraacetic acid (EDTA) or 1-hydroxyethane-1,1-diphosphonic acid (HEDP; the salt is called etidronate) as chemical aids in root canal irrigation (Biel *et al.* 2017, Tartari *et al.* 2017). Whilst EDTA solutions are used by many practitioners (Willershausen *et al.* 2015), a HEDP-based product has not been available on the dental market until very recently (Zollinger *et al.* 2018). HEDP is a non-nitrogenous bisphosphonate, which is widely used in food disinfection, soaps, water cleansing, and dishwasher tablets (Becker 2016). As a tetraprotic acid (Fig. 1) with the acidity of the first proton being rather strong, HEDP has four pK_a values, from pK_{a1} 1.35 to pK_{a4} 11.3 (O'Neil 2006). Both, HEDP and EDTA, exist as disodium (Na_2) and tetrasodium (Na_4) salts, which can be dissolved in water to form aqueous irrigants of different pH (O'Connell *et al.* 2000, Zehnder *et al.* 2005b). Na_2EDTA is not water-soluble unless the pH of the solution is adjusted by adding lye (NaOH). It is thus easier to use the tetrasodium salt, which readily dissolves in pure water (O'Connell *et al.* 2000), and has a dissolution maximum of 45 wt% (Biel *et al.* 2017). The tetrasodium EDTA induces a highly alkaline pH, while the standard EDTA solutions used in endodontics are based on Na_2EDTA and feature a pH in the moderately alkaline range (Tartari *et al.* 2017). The tetrasodium salt of HEDP, i.e. the tetrasodium etidronate, has an NaOCl compatibility that is even higher than that of Na_4EDTA, so that this salt can be directly dissolved in a NaOCl solution, which then remains useful for at least 1 h (Biel *et al.* 2017, Zollinger et al. 2017). Earlier studies, however, have hinted at the possibility that smear layer removal by EDTA could be influenced by pH, with alkaline solutions showing a weaker effect (Tartari *et al.* 2017, Tartari *et al.* 2018). To our knowledge, no such data exist for HEDP.

The goal of the current study was to compare the effect of pH on the decalcifying effect of EDTA and HEDP on human dentine disks covered by a smear layer. Calcium values in solutions that contained these specimens were measured, and the area of visually opened tubules was calculated. An attempt was made to reduce variance in the latter outcome by relating the area of apparently opened tubules after exposure to test and control solutions to that after complete smear layer removal, so that each disk could serve as its own control.

Materials and methods

Preparation of dentine disks with standardized smear layer

Treatment and evaluation steps are summarized in Fig. 2. Fifty non-carious human third molars extracted for reasons not related to this study were used. Informed written consent was obtained from all donors that their teeth could be used for bench-top studies. Because teeth were anonymized, e.g. could not be traced back to their donors, this investigation was exempt from the need to get ethical approval by the local ethics committee (Federal Act on Research involving Human Beings). After extraction, tissue remnants were removed using a curette. All necessary precautions were taken to protect personnel handling these teeth from infection. To sterilize the teeth, they were immersed in 1% NaOCl (Laboratorium Dr. G. Bichsel, Interlaken, Switzerland) and sonicated in a water bath (Benzer Dental AG, Zurich, Switzerland) for 5 min. Subsequently, teeth were stored in phosphate-buffered saline (PBS, Thermo Fisher Scientific, Waltham, MA) at 4°C. To cut the dentine disks, the apical half of each root was fixated on an electron microscopy stub (Wenka, Karl Wenger, Courgenay, Switzerland) using self-curing acrylic resin (Paladur, Heraeus Kulzer, Hanau, Germany). Subsequently, a 2-mm root cross-section was cut from each tooth directly below the enamel-cementum junction using a saw microtome (Leica, Modell SP1600, Nussloch, Germany) under constant water-cooling. This method results in disks with exposed dentinal tubules on their coronal aspect, while at the same time the pulp space is not exposed (De-Deus *et al.* 2006). Cementum was removed from the lateral aspects of the disks using a diamond bur (80-µm cylindrical diamond bur, Intensiv, Montagnola, Switzerland) in a counter-angle hand piece. The coronal surface of the disks was marked at the edge using this bur. Disks were then immersed in PBS at 4°C until further use. As assessed in a pilot study and shown on the specimens treated with PBS, the cutting of the disks as described above resulted in a homogeneous smear layer covering the dentinal tubules. Hence, no further steps were taken to create any additional dentine fillings.

Solutions

Test solutions were prepared from pure chemicals. The EDTA powders were bought from Merck (Darmstadt, Germany). The HEDP (etidronate) powders were acquired from Zschimmer und Schwarz (Burgstädt, Germany). The 17% Na_2EDTA solution, which is the standard used for

endodontic irrigation, served as a reference. To compare the decalcifying effect of the different molecules, molarity of the other test solutions was adjusted to that of 17% Na_2EDTA, i.e. 0.5 M (Table 1). The pH of these solutions was measured using a calibrated microelectrode (827 pH lab; Metrohm, Herisau, Switzerland).

Treatment of disks

To pre-condition the dentine as is done in clinics in the standard irrigation protocol, disks were immersed in 1% NaOCl for 5 min under constant agitation (100 rpm, Sea Star, Heathrow Scientific, IL, USA), followed by a rinse with deionized water for 10 s. Subsequently, disks were randomly allocated into the 5 groups (n = 10). Disks were kept in 5 mL of PBS in individual polyethylene tubes (Semadeni, Ostermundigen, Switzerland).

For the treatment, disks were transferred to 12-mL polyethylene vials (Semadeni), each. They were immersed in 5 mL of test or control solutions for 1 min under agitation (100 rpm) on an orbital shaker (Sea Star). Subsequently, disks were washed using deionized water and returned to their storage vials filled with fresh PBS. Solutions contained in the test vials were frozen at -20°C until atomic absorption spectroscopy (AAS) analysis. Specimens were then stored at 4°C in PBS until optical analysis, which was performed within the 2 subsequent weeks.

Atomic absorption spectroscopy (AAS)

Test and control solutions that had contained the disks were analyzed for their calcium content using AAS (Model contrAA 300, Analytik Jena, Jena, Germany) with an air-acetylene flame. Measurements were performed in duplicate. As an internal control, solutions were spiked with 10 ppm Ca^{2+} (Certipur, Merck). Measurements were obtained against a standard dilution series of $Ca(NO_3)_2$ in HNO_3 (Certipur). Phosphate was masked using strontium chloride hexahydrate (Emsure, Merck).

Laser microscopy

The coronal surface of each disk (as marked by a bur) was assessed with a 3D laser scanning microscope (VK-X200, Keyence International, Mechelen, Belgium) using the VK Viewer acquisition software by an operator who was blinded to the group assignments. Samples were removed from the storage vials, carefully blotted dry with precision wipes and air-dried for 5 minutes. Five random areas per disk were acquired using a 50× objective and z-stacking with a

z-pitch of 0.1 μm. Images were electronically stored in Tagged Image File (TIF) format for further analysis. After the procedure, disks were again returned to their storage vials containing PBS.

Further demineralization and image analysis

Since dentine is a heterogeneous tissue and variance in its appearance is high even in such standardized samples, an attempt was made here to use each disk as its own control. To this end, disks were demineralized each for 2 extra min in 10% citric acid (Merck) under constant agitation as described above. This treatment opens a maximum amount of dentinal tubules (Reis *et al.* 2008). A pilot study showed that after 2 min, the apparently open area as assed by laser scanning microscopy reaches a peak, and subsequently increases at a much slower rate caused by erosion vs. opening of smear-covered tubules (De-Deus *et al.* 2008). Disks were re-assessed as described above by a blinded operator (5 random images per disk).

For the image analysis, 2 observers (blinded again to group designation) checked all the images and discarded those that were not representative of the specific disk (outliers) or of inferior quality. Subsequently, the images were opened in the ImageJ program (Version 2.0.0-rc-43&1.51h, NIH, Bethesda, MD, USA). The scale was set so that 3.505 pixels correlated to 1 μm. Data was converted to 8-bit gray scale values. On that scale from 0 to 255, pixels with a value below 38 were identified as open tubular area (Fig. 3). Subsequently, the total area of apparently open tubules was calculated. Water artifacts were eliminated by marking and converting into gray areas in the ImageJ program.

Data presentation and analysis

Total calcium that was detected in the test and control solutions after the dentine disks had been immersed for 1 min is expressed as ppm. The total area of open tubules per image (average of 5 per disk, 10 disks per treatment) is presented as μm^2. Relative open dentinal tubule areas as compared to the final treatment with citric acid are presented as per cent (%) values.

Ca^{2+} ppm values in the solutions were evenly distributed within treatment groups, while open tubule area values (both absolute and in relation to the final treatment with citric acid) were not (Shapiro-Wilk test). Consequently, Ca^{2+} ppm values were compared between groups by one-way ANOVA with post-hoc Tukey HSD test. Open tubular areas were compared between groups by Kruskal-Wallis analysis of variance, followed by Wilcoxon signed rank test of each pair with

Bonferroni's correction for multiple testing. The alpha-type error for all these tests was set at 5% ($P < 0.05$). The correlations between Ca^{2+} ppm values in the solution and open tubule areas per individual dentine disk were assessed by calculating Spearman's rho.

Results

The 0.5-M chelator solutions under investigation took up different amounts of calcium: the Na_2EDTA at pH 8.5 was the strongest decalcifying agent, followed by Na_4EDTA (pH 11.4) and Na_2HEDP (pH 4.6), which were statistically similar ($P > 0.05$). The statistically weakest chelator was Na_4HEDP, which had a pH of 11.3 (Fig. 4). PBS did not take up any calcium ($P < 0.05$ compared to all other groups).

Smear layer removal values as assessed by the total amount of apparently open tubules on laser scanning microscopy scans showed a similar picture (Fig. 3). Na_2EDTA opened the highest tubular area ($P < 0.05$ compared to all other groups), followed by the other chelators/pH levels under investigation, which did not differ among each other, but were statistically different from the PBS control group ($P < 0.05$). When the tubular area that was opened by the solutions under investigation was related to the total area of tubules that were opened by a subsequent exposure of the dentine disks to 10% citric acid, the result was similar, with identical statistical groups (Table 2). However, variance in the outcome variable was not reduced by this usage of each individual disk as its own control.

There was a very high correlation between the apparent tubular area that was opened by the solutions and their decalcifying effect assessed by AAS (Spearman's rho = 0.81, P ($P < 0.001$).

Discussion

This study showed that dentine decalcification and smear layer removal by EDTA and HEDP are influenced by the pH of the aqueous solution. EDTA was the stronger chelator than HEDP, and an alkaline pH in solution caused by the extra sodium ions contained in the tetrasodium salts decreased the decalcifying effect of both molecules under investigation as compared to their disodium formulations.

To study smear layer removal by endodontic irrigants on human dentine is tricky and full of methodological pitfalls (De-Deus *et al.* 2011). Therefore, in the current study, a double approach was applied: a robust chemical method in the form of atomic absorption spectroscopy (AAS) was combined with an optical measurement: the area of opened dentinal tubules. This procedure including the proper controls (mere treatment by PBS) allowed sound comparisons between treatments as well as the methods themselves. AAS is a robust analytical method to detect total calcium in solution. To double-check the method and its independence from other dissolved components, we spiked the test solutions in this study with extra calcium; recovery was 99%. Studies on the appearance of instrumented root canal walls on images, on the other hand, are less reliable because of the high heterogeneity of root dentine (Lottanti *et al.* 2009). To avoid this, standardized cross-sections obtained from the coronal roots of human third molars have been used in conjunction with co-site optical microscopy (De-Deus *et al.* 2007). However, variance in the amount of open tubules was still high between specimens, so that further attempts aimed at comparing open tubules within the same dentine disk *(*De-Deus *et al.* 2008). This is possible when merely two decalcifying agents are compared but becomes difficult if multiple comparisons are to be made. Hence, in the current study, we compared opened tubules in standardized dentine disks, and attempted to identify the amount of tubules available in each disk by a final decalcification with citric acid, which is a stronger decalcifying agent than any of the test substances under investigation (Zehnder *et al.* 2005a, De-Deus *et al.* 2006). However, as can be appreciated in Table 2, this addition of an internal reference did not reduce variance in the outcome (open tubular area relative to complete smear layer removal by citric acid). This can be explained by the heterogeneity of the dentine within the same specimen, which appears to be as high as that between such specimens as described in this study. Hence, subtle differences in the effectiveness of two decalcifying agents, such as e.g. between Na_4EDTA and Na_4HEDP may not be picked up by this analysis. A 3D laser scanning microscope was used to acquire images of the experimental dentine samples. Studies showing dentine surface topographies frequently involve scanning electron microscope (SEM) assessments of treated surfaces. However, a SEM requires dried samples due to the high vacuum that is required therein. Thus, the samples have to undergo a dehydration process and the whole image acquisition is prolonged. Moreover, longitudinal assessment as done here are not possible when a SEM is used for analysis (De-Deus *et al.* 2011). A normal light microscope could be used at ambient conditions but it lacks the depth of focus for rough and wet samples, as it is the case for dentine samples. Therefore, the 3D laser scanning

microscope offered the advantage of easy sample handling at ambient conditions while obtaining high resolution images of the desired magnification, especially when using the so-called z-stacking of several layers of images.

The differences between the two species, namely EDTA and HEDP are the complex constants to bind the Ca^{2+} ion. EDTA forms a more stable complex than HEDP and, thus, it can bind more calcium (Fig. 4; (Stanga 2010)). Comparing the corresponding salts of the different chelators, di- vs. tetrasodium salt, the presence of more metal ions of the tetrasodium salt can possibly influence the chelation potential of the sequestrant, with less chelation for Na_4. Next to that fact, the disodium salt solutions have a lower pH compared to the tetrasodium salts. This lower pH could have had an effect on the calcium dissolution from dentine discs, too. These results are in line with published data on Na_3- vs Na_4EDTA (Tartari *et al.* 2017, Tartari *et al.* 2018).
The current study confirms previous investigations in that the decalcifying effect of solutions is congruent with their effectiveness in removing smear layer (Zehnder *et al.* 2005b). The optical assessment of dentine discs is cumbersome and time-consuming. Hence, in future investigations on the comparative effectiveness of such agents, AAS analysis may be sufficient to make these comparisons.

Conclusion

Calcium chelation and thus smear layer removal by EDTA and HEDP irrigants are influenced by the amount of sodium contained in the salts to prepare the respective solutions and thus pH. High-pH solutions containing these chemicals remove less calcium and smear layer from root dentine than more neutral or acidic counterparts. EDTA is a more potent calcium sequestrant than HEDP.

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Conflict of interest

The authors have stated explicitly that there is no conflict of interests in connection with this article

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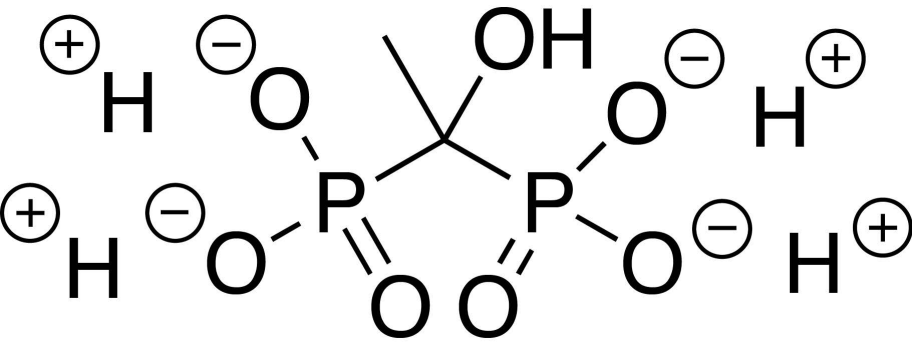
Captions:

Figure 1 Structure of the protonated species of 1-hydroxyethane-1,1-diphosphonic acid (HEDP). The reported pK_a values correspond to the respective dissociations of the various protons and describe the acidic strength (from lower to higher pK_a value).

Figure 2 Flow chart depicting the preparation, treatment, and evaluation steps performed in the current study.

Figure 3 Laser microscopy images of the standardized dentine disks after immersion in test or control solutions. Upper lane: original images in 8-bit gray scale. Pixels with a value below 38/255 were identified as open tubular area and exported (lower lane). Areas of apparently open tubules were then calculated using the ImageJ program.

Figure 4 Bar chart depicting the amount of calcium that was chelated by the different solutions. Error bars indicate standard deviations, identical letters that there was no significant difference between data sets at the 5% level (one-way ANOVA with post-hoc Tukey HSD test).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OH
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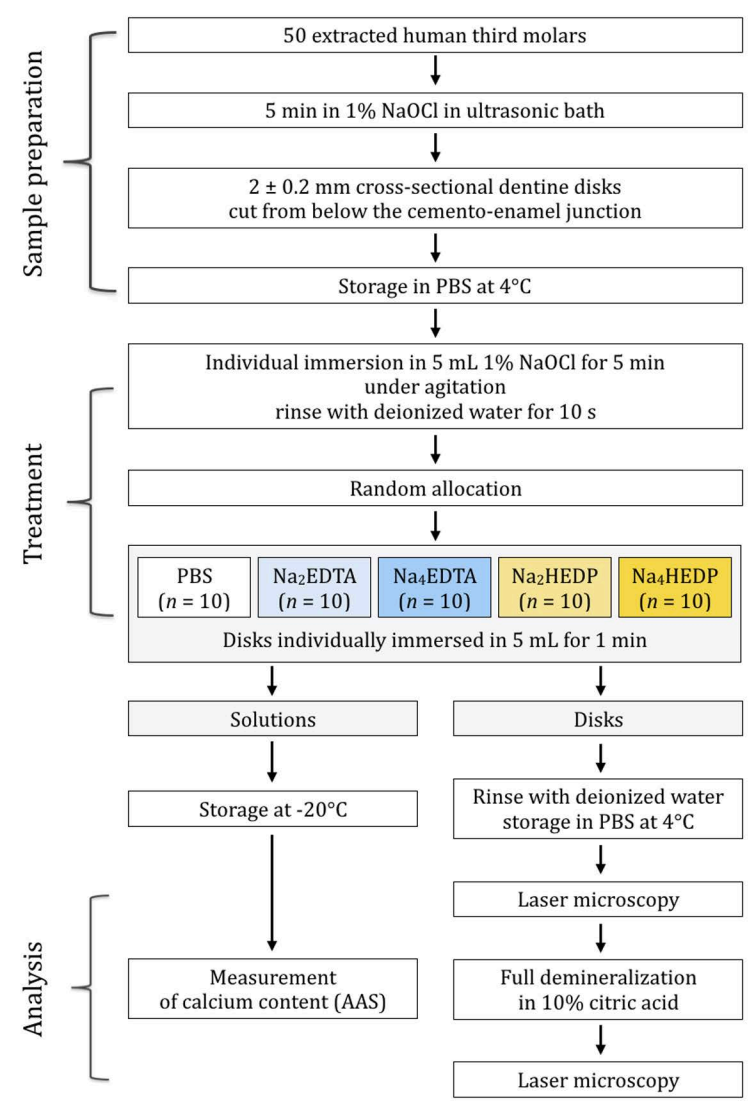
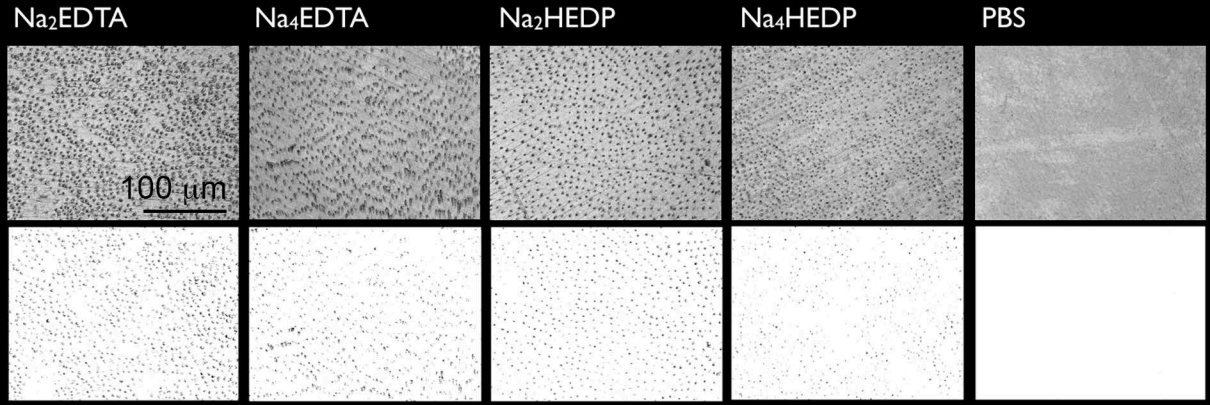

Sample preparation
50 extracted human third molars
5 min in 1% NaOCl in ultrasonic bath
2 ± 0.2 mm cross-sectional dentine disks
cut from below the cemento-enamel junction
Storage in PBS at 4°C
Treatment
Individual immersion in 5 mL 1% NaOCl for 5 min
under agitation
rinse with deionized water for 10 s
Random allocation
PBS
(n = 10)
Na2EDTA
(n = 10)
Na4EDTA
(n = 10)
Na2HEDP
(n = 10)
Na4HEDP
(n = 10)
Disks individually immersed in 5 mL for 1 min
Solutions
Disks
Storage at -20°C
Rinse with deionized water
storage in PBS at 4°C
Laser microscopy
Analysis
Measurement
of calcium content (AAS)
Full demineralization
in 10% citric acid
Laser microscopy


Na_2EDTA
Na_4EDTA
Na_2HEDP
Na_4HEDP
PBS
100 μm

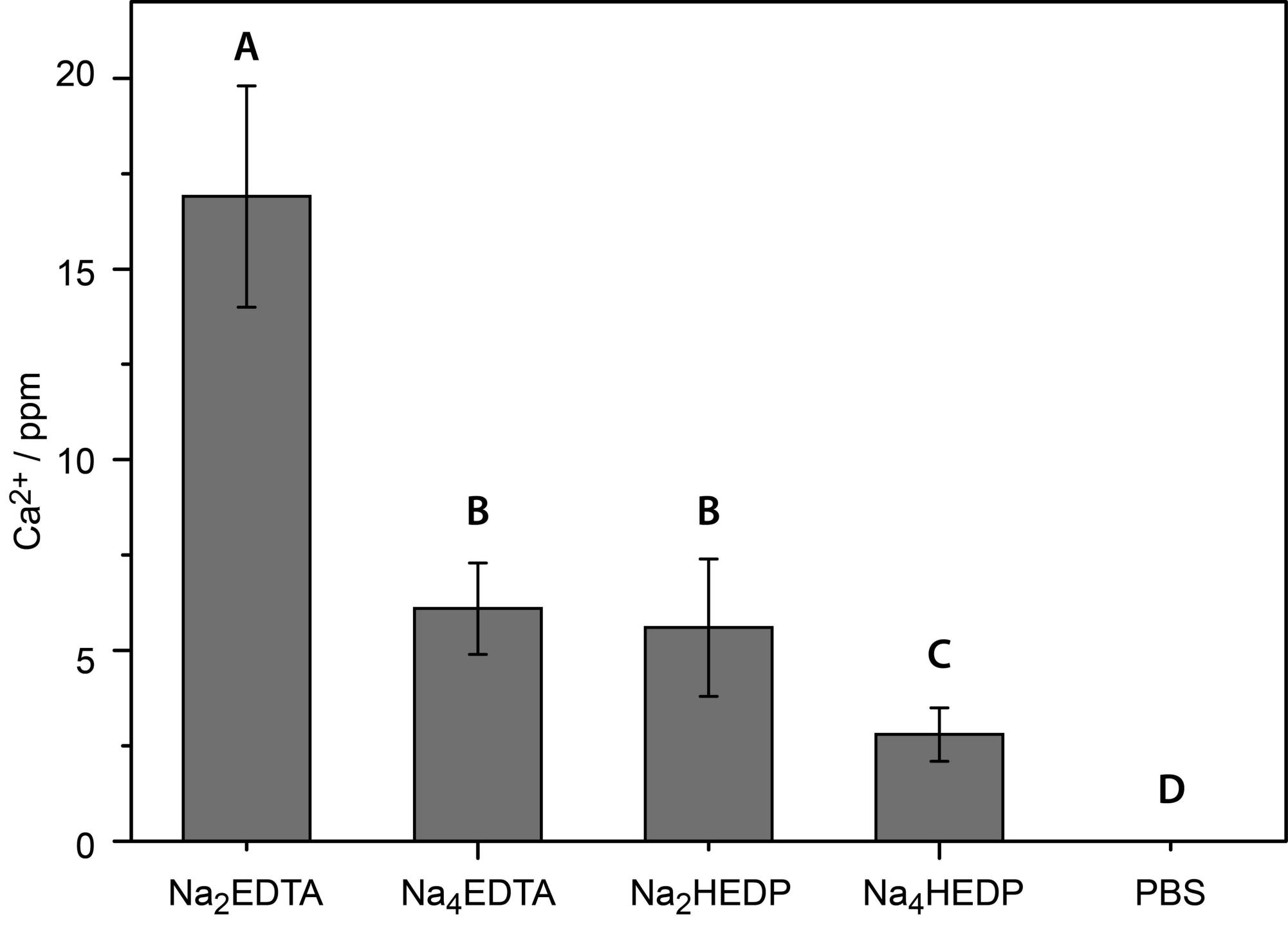

A
B
B
C
D
20
15
10
5
0
Ca2+ / ppm
Na2EDTA
Na4EDTA
Na2HEDP
Na4HEDP
PBS

Table 1 Chelator solutions used in this study adjusted to the molarity of 17% Na_2EDTA

Salt	MW (g/mol)	Wt/vol	Mol/L	pH
Na_2EDTA	336.2	17%	0.5	8.5*
Na_4EDTA	380.2	19%	0.5	11.4
Na_2HEDP	248.0	12%	0.5	4.6
Na_4HEDP	298.0	15%	0.5	11.3

*A 17% Na_2EDTA solution was used as the reference, its pH was adjusted to 8.5 using NaOH so that the EDTA dissolved. All other salts were merely dissolved in deionized water;
MW : molecular weight of the molecule;
Wt/vol indicates the weight of the salt per volume of liquid of the dissolved salt and the water.

Table 2 ImageJ measurements of apparently open tubules on dentin disks after immersion in solution (1 min) followed by citric acid (2 min); medians and inter-quartile ranges

Solution	After immersion (μm^2)	After citric acid (μm^2)	Relative area (%)
PBS	27 (22) ^{A}	4151 (9046)	0.7 (1.3) ^{a}
Na_2EDTA	2047 (1032) ^{C}	2761 (1402)	74.2 (95.3) ^{c}
Na_4EDTA	581 (692) ^{B}	3637 (2847)	15.2 (28.0) ^{b}
Na_2HEDP	953 (722) ^{BC}	3378 (3059)	28.2 (13.9) ^{bc}
Na_4HEDP	364 (255) ^{B}	3282 (1987)	9.5 (18.3) ^{b}
P-value*	< 0.001	0.3	< 0.001

*Kruskal-Wallis test was used to assess whether the variable «Solution» had a significant impact on the outcome measures that are listed. Shared superscript letters indicate that there was no significant difference at the 5% level between solutions regarding an outcome (Wilcoxon signed rank test, Bonferroni's adjustment for multiple comparisons, i.e. the *P*-value had to be below 0.005 for the individual comparisons).